

ORIGINAL ARTICLE

MA026, an anti-hepatitis C virus compound, opens tight junctions of the epithelial cell membrane

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MA026 is an antiviral natural compound against hepatitis C virus (HCV). It was recently reported that MA026 binds claudin-1 (CLDN1) and inhibits HCV infection. Although CLDN1 is an important component of tight junctions (TJ) in the epithelial cell layer, the effects of MA026 on the TJ barrier function remained to be revealed. Here we report that MA026 irreversibly opens the TJ. MA026 irreversibly increased FD4 permeability and decreased transepithelial electrical resistance (TER) for at least 5 h. Although MA026 increased Ca²⁺ influx in layered MDCKII cells, the Ca²⁺ influx was less than that of capsaicin, a reversible TJ opener. Moreover, MA026 did not induce the dephosphorylation of cofilin and reorganization of F-actin structure. Although the mechanism is left to be disclosed, these results suggest that MA026 is a novel irreversible TJ opener probably by targeting CLDN1.

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INTRODUCTION

MA026 (Figure 1a) is a lipocyclodepsipeptide isolated from the fermentation broth of *Pseudomonas* sp. RtIB026.¹ This microorganism was isolated from the digestive tract of rainbow trout (*Oncorhynchus mykiss*) with resistance to the infectious hematopoietic necrosis virus (IHNV), a member of the genus *Novirhabdovirus* in the *Rhabdoviridae* family. MA026 shows the antiviral activity against not only IHNV, but also hepatitis C virus (HCV), a member of *Flaviviridae* family. Because IHNV and HCV belong to different families, it was suggested that MA026 shows antiviral activity by targeting either the conserved protein between the virus families, or the host-cell proteins.

Recently, we reported the first total synthesis of MA026 and proposed that the synthesized MA026 inhibited the HCV entry process into a hepatocyte cell line.² In addition, we revealed that MA026 bound with the VFDSL peptide, the sequence of which is a part of claudin-1 (CLDN1). Because it has been reported that CLDN1 is highly expressed in hepatocytes and has an important role during the post-cell binding process of HCV entry,³ these results strongly suggest that MA026 binds to CLDN1 and inhibits HCV entry. However, the other effects of MA026 on the CLDN1 function remain to be revealed.

Claudins are the most important component of tight junctions (TJ). TJ are structures in polarized epithelial cells that restrict the paracellular movement of solutes and macromolecules across

epithelia.⁴ TJ are composed of a complex combination of transmembrane integral proteins (occludin and tricellulin, as well as claudins), along with several intracellular proteins such as ZO-1, which connects the transmembrane proteins to the actin cytoskeleton.^{5,6} Studies using CLDN1-knockout mice have indicated that CLDN1 is indispensable after birth and necessary for the epidermal barrier.⁷ Because MA026 binds to recombinant CLDN1 protein, there is a possibility that MA026 impairs the barrier functions of TJ. Here we investigated the effects of MA026 on the TJ opening, and found that MA026 opens TJ irreversibly by a mechanism different from that of capsaicin.

MATERIALS AND METHODS

Compounds, cell culture and cell viability measurement

MA026 was prepared as described in previous reports.^{1,2} Capsaicin, ionomycin, and latrunculin A (LatA) were purchased from Sigma (St. Louis, MO, USA).

MDCKII cells were grown in DMEM (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% FBS (Nichirei Biosciences Inc., Tokyo, Japan) and 1% penicillin-streptomycin (Nacalai Tesque Inc.) in a humidified atmosphere containing 5% CO₂.

For cell viability measurement, MDCKII cells were seeded in 96-well plate (Iwaki, Tokyo, Japan) at a density of 3.4 × 10⁴ cells per well. The cells were cultured for 3 days to establish monolayer integrity. Cells were treated with several concentrations of MA026. After 4 h, 10 μl cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added to each well. The plate was incubated at 37 °C. Absorbance at 450 nm was measured by iMark

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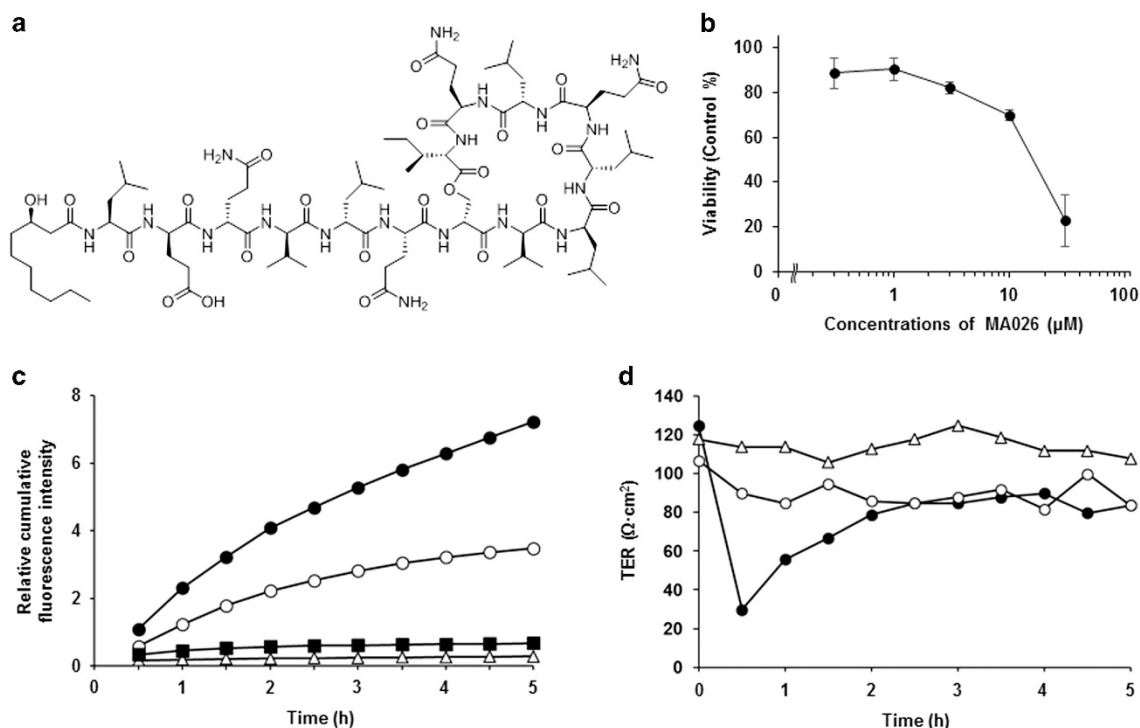
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Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the IC_{50} value was calculated.

Transport and intracellular Ca^{2+} measurements

MDCKII monolayer permeability was measured as previously described.⁸

For intracellular Ca^{2+} measurement, MDCKII cells were seeded in 96-well plates with a clear bottom and black wall (Greiner Bio-One, Frickenhausen, Germany) at a density of 3.4×10^4 cells per well. The cells were cultured for 3 days, and the medium was changed every day to establish monolayer integrity. After specific treatments, the monolayers were washed with Hank's balanced salt solution (HBSS), then treated with fluo-8 loading buffer (5 μM Fluo-8 (AT Bioquest Inc., Sunnyvale, CA, USA), 2.5 mM probenecid (Enzo Life Sciences Inc., Farmingdale, NY, USA), 1% F-127 (Biotium Inc., Fremont, CA, USA)) in HBSS at 37 °C for 1 h. Cells were washed with HBSS twice and then the recording buffer (2.5 mM probenecid in HBSS) and various compounds were added. Fluo-8 fluorescence was measured every 3 min for 60 min with a PowerscanHT fluorescence microplate reader (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) at excitation and emission wavelengths of 485 and 528 nm, respectively.

TER Measurements

For the TER experiments, MDCKII cells were seeded in 6.5 mm diameter transwells (pore size 0.4 mm, Corning Inc., Corning, NY) coated with collagen at a density of 4.0×10^4 cells per well. The cells were cultured for 3 days to establish monolayer integrity. TER was measured as previously described.⁸

Immunoblotting and F-actin staining

MDCKII cells were seeded in a 24-well plate (Iwaki, Tokyo, Japan) at a density of 2.0×10^5 cells per well. The cells were cultured for 3 days and the medium was changed every day to establish monolayer integrity. After specific treatments, the monolayers were washed once with PBS and lysed with lysis

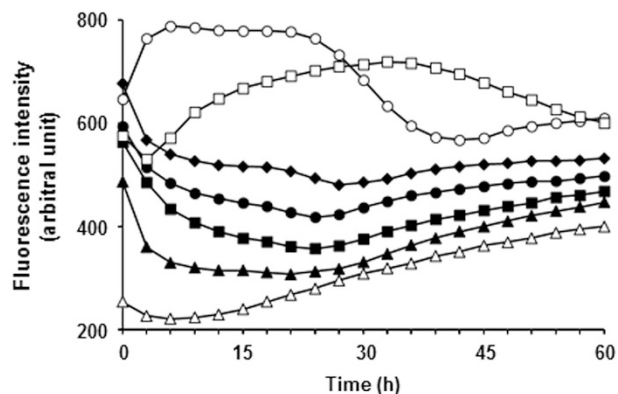


Figure 2 MAO26 weakly induced Ca^{2+} influx in the MDCKII cell monolayer. MAO26 induced Ca^{2+} influx in the MDCKII monolayer. The final concentrations were 0 μM (Δ), 0.1 μM (\blacktriangle), 1 μM (\blacksquare), 3 μM (\bullet) and 10 μM (\blacklozenge) of MAO26, 10 μM ionomycin (\circ), and 300 μM capsaicin (\square). Representative data of 2 independent experiments are shown.

buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol and 5% β -mercaptoethanol, pH 6.8). After sonication, the cell extracts were boiled at 65 °C for 10 min, separated by SDS-PAGE, transferred to a polyvinylidene fluoride microporous membrane (Millipore, Billerica, MA, USA), blocked with 5% skimmed milk (Megmilk Snowbrand Co., Ltd., Sapporo, Japan) or setsuyakukun supporter (DRC Inc., Tokyo Japan), probed with the appropriate primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (KPL, Gaithersburg, MD, USA), and detected by enhanced chemiluminescence (Nakalai Tesque). Anti-claudin-1 (No.13255) was from Cell Signaling Technology (Beverly, MA, USA). Anti-actin (20–33) (No. A5060) and anti-phospho-

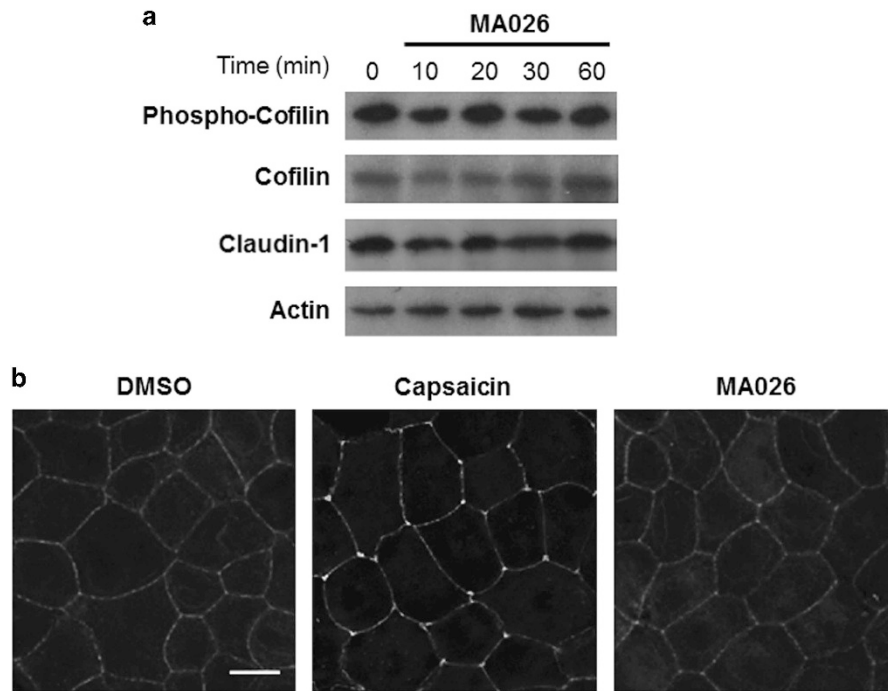


Figure 3 MA026 did not induce notable change in the phosphorylation of cofilin, the amount of CLDN1 protein, and F-actin organization. (a) after treatment of 3 μM MA026 for the indicated durations, MDCKII monolayers were lysed and subjected to western blot analysis. The representative data of two independent experiments are shown. (b) MDCKII monolayers were treated with DMSO control, 300 μM capsaicin, or 3 μM MA026 for 45 min, and stained with CF488A-phalloidin. Each of z-stack images was deconvoluted and representative views were shown. Bar: 10 μm .

cofilin (Ser 3, #sc-21867-R) were from Sigma and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

For F-actin staining, MDCKII monolayers grown on LABTEK chamber Permanox slides (Nunc, Rochester, NY, USA) were washed with PBS, fixed for 10 min with 3.7% formaldehyde, and permeabilized for 5 min in PBS containing 0.2% Triton X-100. After blocking with PBS containing 5% skimmed milk for 30 min at 37 $^{\circ}\text{C}$, CF488A-phalloidin was applied and incubated for 1 h at 37 $^{\circ}\text{C}$. Fluorescence images were acquired with a Leica AF6000 deconvolution microscope system equipped with a fully automated microscope (DMI6000B) and a DFC350 FX digital camera (Leica Microsystems, Heidelberg, Germany). The z-stacks were collected at 0.2 μm intervals. Image stacks were deconvoluted using Leica LAS AF6000 software.

RESULTS AND DISCUSSION

Before investigating the effects of MA026 on TJ function, we examined the cytotoxicity of MA026 on MDCKII monolayers. The IC_{50} value of MA026 was calculated as 13.4 μM , and 4 h treatment with MA026 at a concentration of 3 μM did not show notable cytotoxicity to the monolayers (Figure 1b). Therefore, we used a concentration of 3 μM MA026 or less for the subsequent experiments. We next examined whether MA026 could increase TJ permeability. Fluorescein isothiocyanate-conjugated dextran-4 (FD4) solution was applied to the apical side of the MDCKII monolayer in transwells, and then several concentrations of MA026 were added. Every 30 min, the basolateral solution was collected to measure the amount of permeated FD4 (Figure 1c). Latrunculin A (LatA), an irreversible TJ opener, was found to increase FD4 permeability continuously.⁸ Like LatA, MA026 increased FD4 permeability from 30 min to 5 h continuously, suggesting an irreversible TJ opening. To determine the degree of TJ tightness, we next measured the transepithelial electrical resistance (TER, Figure 1d). LatA decreased the TER continuously as previously reported.⁸ Interestingly, MA026 drastically decreased TER within 30 min. Although this decrease was gradually recovered,

the TER values were the same as that of LatA at least 5 h. Taken together, these results indicate that MA026 is an irreversible TJ opener.

We previously demonstrated that capsaicin induces reversible TJ opening via Ca^{2+} influx.^{8–10} Therefore, we investigated the ability of MA026 to induce Ca^{2+} influx in a MDCKII monolayer (Figure 2). The positive control ionomycin immediately increased the cellular Ca^{2+} concentration, and beginning at around 20 min the concentration was attenuated (Figure 2, open circles). Capsaicin-induced Ca^{2+} influx peaked after 30 min and continued for at least 60 min (Figure 2, open squares). In contrast, MA026 induced a Ca^{2+} influx peak just after the measurement started, and then sustained this level for a longer duration (Figure 2, closed symbols). Ca^{2+} influx by MA026 was detectable from 0.1 μM and increased dose-dependently, although the Ca^{2+} influx by MA026 was lower than that by capsaicin.

Finally, we analyzed the perturbation of TJ-related proteins. We focused on the phosphorylation of cofilin, reorganization of F-actin, and the amount of CLDN1, because cofilin dephosphorylation (activation) and subsequent reorganization of F-actin are the critical signals for TJ opening induced by capsaicin, and CLDN1 has been suggested to be a target molecule of MA026. We have shown that phosphorylation of cofilin is abolished as quickly as 10 min after capsaicin treatment, but recovers at around 60 min.⁸ In contrast, 3 μM MA026 did not induce notable change in the cofilin phosphorylation level until 60 min (Figure 3a); nevertheless, TJ permeability had already started to increase by this time point. We also reported that capsaicin induces the actin reorganization: decreased the level of F-actin at bicellular junctions but increased it at tricellular junctions (Figure 3b, reference 8). However, there is no obvious change of F-actin distribution in MA026-treated cells. These results suggest that TJ opening by MA026 is a Ca^{2+} -influx/cofilin dephosphorylation/F-actin reorganization cascade-independent mechanism. Further, the protein amount of CLDN1 was not affected by MA026 treatment,

suggesting that the degradation of CLDN1 is not involved in MA026-induced TJ opening (Figure 3a). In summary, this is the first report regarding the ability of MA026 to increase paracellular permeability. In a previous report, phage display screening revealed that the MA026-binding peptide sequence was VFDSL, which is conserved in the first extracellular loop of CLDN1.² Then, MA026 was found to bind to recombinant CLDN1 protein *in vitro*. Because CLDN1 is an essential protein in the formation of TJ, there is a possibility that MA026 binding to its extracellular loop disrupts TJ integrity. Therefore, we examined the possibility that MA026 induces TJ opening. Unlike the mode of action of a reversible TJ opener, capsaicin, the permeability increase induced by MA026 was irreversible for at least 5 h. Moreover, MA026 increased Ca²⁺ influx to a lesser extent than capsaicin, and did not induce cofilin dephosphorylation and reorganization of F-actin. Because Ca²⁺ influx, the transient activation of cofilin, and subsequent F-actin reorganization seem to be related to reversible TJ opening,⁸ these results indicated that MA026 increases TJ permeability by a mechanism different from that of capsaicin. Although the protein amount of CLDN1 was not affected for 1 h by treatment with MA026, the interaction between MA026 and the CLDN1 extracellular loop may disrupt the claudin–claudin interactions between adjacent cells and induce TJ opening. We are now conducting investigations into how MA026 affects TJ integrity, especially CLDN1 function.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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